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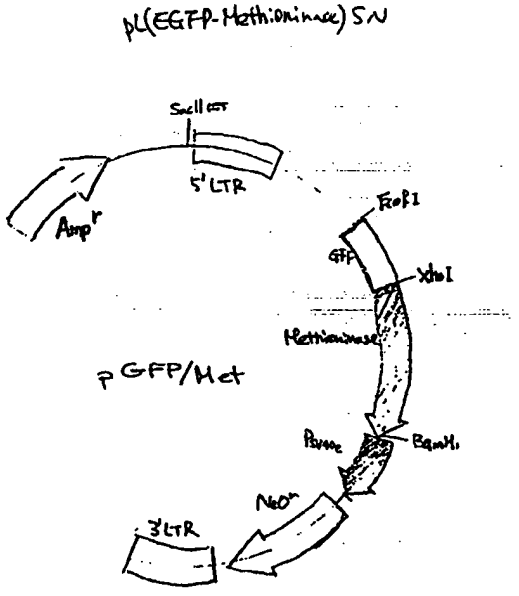
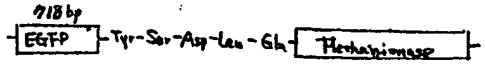
(57) Abstract

A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. The fluorescence can also be monitored by observing the tissues *in situ*. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis. In addition, subjects already harboring tumors can be treated so as to modify the endogenous tumors to contain GFP. This permits clinical applications. Finally, by injecting a contrast dye into a subject harboring a GFP–labeled tumor, angiogenesis in the tumor can be observed directly.





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METASTASIS MODELS USING GREEN FLUORESCENT PROTEIN (GFP) AS A MARKER

Technical Field

The invention relates to the study of tumor progression. Specifically, it concerns model systems for studying the metastasis of tumors in vertebrate systems.

Background Art

It has long been recognized that the ability of tumor tissues to metastasize constitutes a major portion of the life-threatening aspects of malignancy. Metastasis is the growth of secondary tumors at sites different from the primary tumor. Thus, despite surgical removal of the primary tumor, it may not be possible to arrest the progress of this condition. An understanding of the mechanism whereby metastasis occurs will be crucial to the development of protocols whereby the growth of secondary tumors can be controlled. In order to understand the mechanism of metastasis, it will be necessary to provide a model which permits identification of small numbers of tumor cells against a background of many host cells so that secondary tumor emboli and micrometastases can be observed over the course of real time.

Others have demonstrated extravasation and initial seeding steps in tumor metastasis *in vitro* using externally fluorescently labeled tumor cells. Khokha, R. *et al., Cancer Metastasis Rev* (1995) 14:279-301; Koop, S. *et al., Cancer Res* (1995) 55:2520-2523. Further, Margolis, L.B. *et al., In Vitro Cell Dev Biol* (1995) 31:221-226 was able to visualize the migration of externally fluorescently labeled lung tumor cells in host mouse lung in histoculture. In all cases, however, long-term observation was not possible due to the limitation of exogenous fluorescent labels. Retroviral transfer of a green fluorescent protein (GFP) gene has been shown to result in stable transfectants of human cancer cells *in vitro* (Levy, J.P. *et al., Nature Biotechnol* (1996) 14:610-614), as well as of hematopoietic cells (Grignani, F. *et al. Cancer Res* (1998) 58:14-19 and by Cheng, L. *et al. Gene Therapy* (1997) 4:1013-1022).

Attempts have been made to provide such a model using the β-galactosidase gene as a marker (Lin, W.C. *et al., Cancer Res* (1990) 50:2808-2817; Lin, W.C. *et al., Invasion and Metastasis* (1992) 12:197-209). However, this marker has not proved satisfactory, as fresh or processed tissue cannot be used. The present invention provides a marker which permits visualization of tumor invasion and micrometastasis formation in viable fresh tissue. In addition, by providing suitable contrast media, the method of the invention can be adapted to visualize angiogenesis in established and growing tumors. The methods of the invention can be applied not only to models of tumor growth and metastasis, but, through the use of retroviral vectors, can be employed to obtain clinical data in human subjects bearing tumors.

The present invention utilizes green fluorescent protein (GFP) as a marker. Heterologous expression of this protein, principally to monitor expression of fused DNA, was disclosed in U.S. Patent No. 5,491,084. This document describes the expression of GFP in *E. coli* and *C. elegans* and postulates that cells in general can be modified to express GFP. Such expression, according to this document, permits not only a method to monitor expression of fused DNA, but also a means of monitoring protein localization within the cell.

The aspect of the invention which provides a metastatic model has been reported and described in a series of publications. Chishima, T. *et al. Cancer Research* (1997) 57:2042-2047 describe the construction of a dicistronic vector containing the gene for humanized green fluorescent protein (GFP) and dihydrofolate reductase (DHFR). This vector was transfected into CHO-K1 cells to obtain clone-38. Clone-38 showed stable GFP expression which was maintained in the presence of methotrexate (MTX). Clone-38 cells were injected into mice to obtain tumor fragments which were then implanted by surgical orthotopic implantation (SOI) on the ovarian serosa in nude mice. Metastasis could be followed in this model.

Chishima, T. *et al. Proc Natl Acad Sci USA* (1997) 94:11573-11576 describe the preparation of clone-26 by transfection of Anip 973 human lung adenocarcinoma cells with the codon optimized hGFP-S65T clone obtained from Clontech. Clone-26 was injected intravenously into nude mice and the resulting tumors were followed in histoculture.

Chishima, T. *et al. Clin Exp Metastasis* (1997) 15:547-552 and Chishima, T. *et al. Anticancer Res* (1997) 17:2377-2384 describe similar work with clone-26 wherein the cells were inoculated subcutaneously into nude mice resulting in a visualizable tumor which was then implanted into the visceral pleura of nude mice by SOI. Metastases were observed in this model as well.

Chishima, T. *et al. In Vitro Cell Dev Biol* (1997) 33:745-747 describe histoculture of clone-26 and visualization of growth using the fluorescence emitted by GFP.

The contents of the foregoing publications are incorporated herein by reference.

In addition to the foregoing, Cheng, L., *et al.*, *Gene Therapy* (1997) 4:1013-1022, describe the modification of hematopoietic stem cells using the GFP gene under control of a retroviral promoter. Although the authors state that human stem cells are transfected with this system only with difficulty, by using an enhanced form of the GFP, satisfactory brightness could be achieved.

In addition, Grignani, F., *et al.*, *Cancer Res* (1998) 58:14-19, report the use of a hybrid EBV/retroviral vector expressing GFP to effect high-efficiency gene transfer into human hematopoietic progenitor cells.

Vectors containing various modified forms of GFP to provide various colors are marketed by Clontech. The Clontech vectors intended for mammalian cell expression place the GFP under control of the cytomegalovirus (CMV) promoter.

Disclosure of the Invention

The invention provides models which permit the intimate study of formation of metastases from primary tumors in a realistic and real-time setting. By using green fluorescent protein (GFP) as a stable and readily visualized marker, the progression of such metastasis can be modeled and the mechanism elucidated.

Thus, in one aspect, the invention is directed to a method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain

tumor cells that express GFP and observing the excised tissues for the presence of fluorescence.

In another aspect, the invention is directed to a vertebrate subject which has been modified to contain tumor cells expressing GFP.

In these aspects, the vertebrate subject may constitute a model system, such as an immunocompromised mouse wherein tumor cells or a tumor, modified to express green fluorescent protein has been introduced into the subject. Alternatively, the subject may be a human or other vertebrate which natively contains the tumor, but wherein the tumor has been subjected to viral infection or to transfection with a retroviral vector so as to produce said GFP.

In still other aspects, the invention is directed to tumor cells modified to produce GFP under control of heterologous control elements, to cells that are immortalized to provide stable cell lines as well as comprising visible amounts of GFP, to tissues containing metastatic tumors that produce GFP, and to histocultures of tissues which contain such metastasized tumors.

The invention also includes a method to observe and follow angiogenesis in solid tumors which method comprises (usually) exposing and observing said tumors. The tumors will have been modified to express GFP, and the subject will have been administered a contrast dye to permit this observation.

Brief Description of the Drawings

Figures 1a and 1b show the construction of expression vectors useful in the invention.

Modes of Carrying Out the Invention

The invention provides model systems for the study of the mechanism of metastasis of tumors generally, as well as to study angiogenesis in solid tumors. Advantage is taken of the visible marker green fluorescence protein (GFP) to label the tumor cells so that their migration and colonization in tissues distal to the tumor can be followed as the migration and colonization progresses. Further, by administering

to the subject a contrast dye, such as rhodamine, the growth of blood vessels in solid tumors which have been labeled with GFP can also be observed.

The label used in the various aspects of the invention is green fluorescent protein (GFP). The native gene encoding this protein has been cloned from the bioluminescent jellyfish *Aequorea victoria* (Morin, J. *et al., J Cell Physiol* (1972) 77:313-318). The availability of the gene has made it possible to use GFP as a marker for gene expression. GFP itself is a 283 amino acid protein with a molecular weight of 27 kD. It requires no additional proteins from its native source nor does it require substrates or cofactors available only in its native source in order to fluoresce. (Prasher, D.C. *et al., Gene* (1992) 111:229-233; Yang, F. *et al., Nature Biotechnol* (1996) 14:1252-1256; Cody, C.W. *et al., Biochemistry* (1993) 32:1212-1218.) Mutants of the GFP gene have been found useful to enhance expression and to modify excitation and fluorescence. GFP-S65T (wherein serine at 65 is replaced with threonine) is particularly useful in the invention method and has a single excitation peak at 490 nm. (Heim, R. *et al., Nature* (1995) 373:663-664); U.S. Patent No. 5,625,048. Other mutants have also been disclosed by Delagrade, S. *et al., Biotechnology* (1995) 13:151-154; Cormack, B. *et al., Gene* (1996) 173:33-38 and Cramer, A. *et al. Nature Biotechnol* (1996) 14:315-319. Additional mutants are also disclosed in U.S. Patent No. 5,625,048. By suitable modification, the spectrum of light emitted by the GFP can be altered. Thus, although the term "GFP" is used in the present application, the proteins included within this definition are not necessarily green in appearance. Various forms of GFP exhibit colors other than green and these, too, are included within the definition of "GFP" and are useful in the methods and materials of the invention. In addition, it is noted that green fluorescent proteins falling within the definition of "GFP" herein have been isolated from other organisms, such as the sea pansy, *Renilla reriformis*. Any suitable and convenient form of the GFP gene can be used to modify the tumor cells useful in the models of the invention, and for retroviral transformation of endogenous tumors. The particular humanized hGFP-S65T clone is used in the examples set forth below for illustration.

Techniques for labeling cells in general using GFP are disclosed in U.S. 5,491,084 (*supra*).

In one application, the method of the invention provides a model system for studying the effects of various therapeutic candidate protocols and substances on metastatic growth of tumors.

In general, the model involves modifying a vertebrate, preferably a mammal, so as to contain tumor tissue, wherein the tumor cells have, themselves, been modified to contain an expression system for GFP. The tumor cells may arise from cell lines of the invention wherein tumor cells have been modified to contain expression systems for GFP and SV40 T-antigen. Tumors can be formed in such vertebrate systems by administering the transformed cells containing the GFP expression system and permitting these transformed cells to form tumors. Typically such administration is subcutaneous and the tumors are formed as solid masses. The tumors thus formed can be implanted in any suitable host tissue and allowed to progress, metastasize and develop.

Suitable procedures for growing the initial tumor, thus, involve transcutaneous injection of the tumor cells producing GFP, such as CHO cells, HeLa cells, carcinoma and sarcoma cell lines, well established cell lines such as the human lung adenocarcinoma line Anip 973, as well as GFP-containing human breast cancer lines MDA-MB468 and MDA-MB435; human prostate cancer lines PC3 and DU-145, human glioblastoma line 324, mouse melanoma B16 and others that may become available in the art, including the immortalized cells of the invention. The administered cells will have been modified to contain an expression system for GFP. After administration, solid tumors generally develop, typically at the site of subcutaneous injection. These tumors, which are themselves fluorescent, can then be removed and used for implantation in the model vertebrate.

Techniques for implantation of the solid tumors, now labeled with GFP, into vertebrates include direct implantation by surgical orthotopic implantation (SOI) at the desired site, typically the site from which the tumor cells were derived. Suitable sites include lung, liver, pancreas, stomach, breast, ovary, prostate, bone marrow, brain, and other tissues susceptible to malignancy. Once the solid tumors have been implanted, the vertebrate becomes a model system for studying metastasis. The tumor is thus allowed to progress and develop and the vertebrate is monitored for appearance

of the GFP labeled cells at sites distal from the original implantation site. The monitoring can occur either on the whole vertebrate by opening the animal and observing the organs directly with a fluorescent microscope, or the tissues may be excised and examined microscopically. In some cases the tumors are sufficiently bright that opening the animal is unnecessary -- they can be seen directly through the skin. In any case, as GFP is visible to the naked eye, no development systems to stain the tissue samples are required. Tissue samples are simply properly processed as fresh samples in slices of suitable size, typically 1 mm thick, and placed under a microscope for examination. Even colonies of less than 10 cells are thus visible. A variety of microscopic visualization techniques is known in the art and any appropriate method can be used.

In addition, the development of the tumor can be studied *in vitro* in histological culture. Suitable systems for such study include solid supported cultures such as those maintained on collagen gels and the like.

Suitable vertebrate subjects for use as models are preferably mammalian subjects, most preferably convenient laboratory animals such as rabbits, rats, mice, and the like. For closer analogy to human subjects, primates could also be used. Particularly useful are subjects that are particularly susceptible to tumor development, such as subjects with impaired immune systems, typically nude mice or SCID mice. Any appropriate vertebrate subject can be used, the choice being dictated mainly by convenience and similarity to the system of ultimate interest.

Any suitable expression system operable in the tumor cells to be implanted may be used. A number of vectors are commercially available that will effect expression in tumor cells of various types. The nature of the vector may vary with the nature of the tumor and the vertebrate in which it finds its origin. However, when GFP is used to visualize metastasis in a model system, it is preferred to utilize vectors which do not use retroviral or other viral promoters which may complicate the nature of the model.

In order to provide cell lines that are helpful in establishing tumors for these model systems, it is also advantageous to employ expression vectors which provide the cells with the SV40 T-antigen. The presence of this antigen ensures immortality

of the culture. Thus, particularly useful in the invention are vectors which comprise expression systems that result in the production both of GFP and SV40 T-antigen.

In order to transfect and modify the transformed cells which are effective in generating tumors, any suitable transfection method may be used, such as liposomes, calcium phosphate precipitation, electroporation and use of a gene gun. Lipofection is preferred.

In contrast, when the method of the invention is used to visualize metastasis in tumors that natively occur in a subject such as a human cancer patient, vectors that employ retroviral or other viral promoters are preferred. The use of such vectors permits the insertion of an expression system for GFP into the already existent tumor. In addition, the expression system may contain nucleotide sequence encoding other useful proteins such as therapeutic proteins which permit simultaneous diagnosis of metastasis and treatment. Among such suitable proteins are included methioninase (see, for example, PCT/US93/11311 and PCT/US96/09935). Such proteins may be produced either as fusions with the GFP, or independently either using a dicistronic expression system or independent expression systems, one for the therapeutic protein and the other for the GFP.

Retroviral based expression systems for GFP have already been described by Grignani, F. *et al. Cancer Res* (1998) 58:14-19 and by Cheng, L. *et al. Gene Therapy* (1997) 4:1013-1022. In these reports, the retroviral expression system itself was used to transfect hematopoietic progenitor cells or packaging cells were employed to provide virus-containing supernatants which can be used directly for infection of the mammalian cells. Thus, in the method of the invention, the tumor contained in the vertebrate subject is typically infected with virus which has been modified and packaged to contain the expression system for GFP. *In situ* infection with virus results in the ability of the tumor to produce GFP and, in effect, label itself.

Various retroviral systems useful in producing proteins in mammalian cells are known in the art. Examples include commercially available vector and packaging systems such as those sold by Clontech, San Diego, California, including their Retro-X vectors pLNCX and pLXSN which permit expression of GFP under a variety of promoters by insertion into the multiple cloning site. These vectors contain ψ^* (the

extended viral packaging signal) and antibiotic resistance genes for selection. A number of these systems have been developed for use in gene therapy, including vectors which provide a multiple cloning site sandwiched between 5' and 3' LTR derived from retroviral sources, and thus would be useful in labeling the tumors of human patients.

Thus, retroviral based vectors such as those set forth in Figures 1a-1b can be transfected into packaging cells and transferred directly to targeted cancer cells or supernatants from the packaging cells can be used to infect tumor cells with the retrovirus. Preferred combinations of retrovirus and packaging cells include the GFP-retrovirus vector pLEIN in PT-67 packaging cells. Co-culture of the packaging cells with colon cancer cells results in transfer of the GFP-retrovirus to the cancer cells.

Using histoculture techniques, and supernatants from PT-67 packaging cells generating GFP-pLEIN virus, the successful modification of a human cancer tissue to display the fluorescence associated with GFP has been demonstrated. For use *in vivo*, the virus is administered, preferably locally to the tumor, which can be observed within hours after injection either of packaging cells or of the viral containing supernatants. The malignant cells can be identified by their green color, sometimes sufficiently bright so that the tumors can be seen through the skin.

In addition to direct observation of tumor metastasis and growth either in a model system or in a vertebrate, typically mammalian and more typically a human subject which is already afflicted by a tumor, the methods of the invention can be adapted to observe angiogenesis in solid tumors. The tumor is itself labeled with GFP as described above. The subject is then administered a contrast dye, typically by injection, preferably intravenous injection, which allows blood vessels in the tumor to be observed. Suitable dyes include rodamine and other contrast dyes. Any dye which forms a contrasting color with the green color of the GFP can be used. Preferably, the dye is coupled to an inert polymer such as polyethylene glycol to increase the length of time the dye will remain in the blood vessel. A sufficient amount of dye is provided to permit ready visualization; the amount of dye required will depend on the choice of dye, the location of the tumor, the nature of the background GFP, and the method used for observation. Within a few minutes, vessels growing into the solid

tumors in such areas as the mesentery, colon wall, and omentum can be observed. Observations can be continued over substantial periods; for example, angiogenesis after several hours is still observed by using this method.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Preparation of Tumor Cells that Produce GFP

The humanized hGFP-S65T clone described by Zolotukhin, S. *et al., J Virol* (1996) 70:4646-4654 was used as the green fluorescent protein coding sequence. This codon-optimized gene was purchased from Clontech Laboratories, Inc. (Palo Alto, CA) and ligated into the dicistronic expression vector (pED-mtx[1]) obtained from Genetics Institute, Cambridge, MA and described in Kaufman, R.J. *et al., Nucleic Acids Res* (1991) 19:4485-4490. hGFP-S65T was digested with *Hind*III and blunted; the entire hGFP coding region was excised with *Xba*I and then unidirectionally subcloned into pED-mtx[1] which had been digested with *Pst*I, blunted and then further digested with *Xba*I.

CHO-K1 cells were cultured in DMEM containing 10% fetal calf serum, 2 mM L-glutamine and 100 μM nonessential amino acids. Near confluent cells were incubated with a precipitated mixture of LipofectAMINE™ reagent (GIBCO) and saturating amounts of plasmids for six hours and then replenished with fresh medium. The cells were harvested by trypsin/EDTA 48 hours later and subcultured at 1:15 into selective medium containing 1.5 μM methotrexate (MTX). Cells with stably integrated plasmids were selected in MTX-containing medium and isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by EDTA. After amplification and transfer, Clone-38 was selected because of its high-intensity GFP fluorescence and stability.

In a similar manner, Anip 973 cells, a human lung cancer cell line obtained from Harbin Medical University, China, were cultured as described above for CHO-K1 cells except using RPMI1640 (GIBCO) in place of DMEM. Transfection,

selection and amplification and transfer were conducted as described above. Clone-26 was chosen because of its high-intensity GFP fluorescence and stability.

Example 2

Mouse Model Using Modified CHO Cells

Clone-38, which was stable at 1.5 μM MTX and which proliferated at the same rate as the parental CHO-K1 cells as ascertained by comparing doubling times, was used in this model.

Three six-week old Balb/C nu/nu female mice were injected subcutaneously with a single dose of 10^7 Clone-38 cells that had been harvested by trypsinization and washed three times with cold serum-containing medium and then kept on ice. The cells were injected in a total volume of 0.4 ml within 40 minutes of harvesting and the nude mice sacrificed three weeks after injection. All of the mice had a subcutaneous tumor ranging in diameter from 13.0 mm to 18.5 mm (mean = 15.2 mm ± 2.9 mm). The tumor tissue was strongly fluorescent. It was shown by extracting GFP from cultured Clone-38 cells in comparison to Clone-38 cells prepared from the tumor that the levels of production of GFP were the same in both.

To construct the model, tumor fragments (1 mm^3) derived from the nude mouse subcutaneous Clone-38 tumor grown as described above, were implanted by surgical or surgical orthotopic implantation (SOI) on the ovarian serosa in six nude mice as described by Fu, X. *et al., Anticancer Res* (1993) 13:283-286, incorporated herein by reference. Briefly, the mice were anesthetized by isofluran inhalation and an incision was made through the left lower abdominal pararectal line and peritoneum to expose the left ovary and part of the serosal membrane, which was scraped with a forceps. Four 1 mm^3 tumor pieces were fixed on the scraped site with an 8-0 nylon suture and the ovary then returned to the peritoneal cavity. The abdominal wall and skin were closed with 6-0 silk sutures.

Four weeks later, the mice were sacrificed and lung and various other organs were removed. The fresh samples were sliced at approximately 1 mm thickness and observed directly under fluorescent and confocal microscopy. Samples were also processed for histological examination for fluorescence and conventional staining.

Frozen sections were prepared wherein the slides were rinsed with phosphate buffer saline and fixed for 10 minutes at 4°C; 10% formaldehyde plus 0.2% glutaraldehyde and PBS were added and the slides were then washed with PBS. The fixed tissue was stained with hematoxylin and eosin using standard techniques.

Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, VT). Confocal microscopy was with an MRC-600 Confocal Imaging System (Bio-Rad) mounted on a Nikon microscope with an argon laser.

The mice, at sacrifice, had tumors in the ovaries ranging in diameter from 18.7 mm-25.3 mm (mean 21.9 ± 3.1 mm). The fresh organ tissues examined under fluorescence microscopy with no treatment of the tissues showed seeding of the tumor throughout the peritoneal cavity, including the colon (6/6 mice), cecum (5/6), small intestine (4/6), spleen (1/6), and peritoneal wall (6/6). Numerous micrometastases were detected in the lungs of all mice and multiple micrometastases were also detected on the liver (1/6), kidney (1/6), contralateral ovary (3/6), adrenal gland (2/6), para-aortic lymph node (5/6) and pleural membrane (5/6). Single-cell micrometastases could not be detected by the standard histological techniques described above and even the multiple cell colonies were difficult to detect using them. As the colonies developed, the density of tumor cells decreased markedly in the center.

In an additional experiment, $5x10^6$ Clone-38 cells were injected into a nude mouse through the tail vein and the mouse sacrificed after two minutes. Fresh visceral organs were analyzed by fluorescence microscopy and showed the presence of fluorescent cells in peritoneal wall vessels which formed emboli in the capillaries of the lung, liver, kidney, spleen, ovary, adrenal gland, thyroid gland and brain.

Thus, using these techniques, progression of micrometastasis can be observed as seeded cells develop into colonies within the relevant target organs. Further, screening for micrometastases can be done easily and quickly in all systemic organs.

Example 3

Murine Model Using Human Lung Cancer Cells

The procedures are generally those set forth in Example 2 except that Clone-26 cells as prepared in Example 1 were used instead of Clone-38 CHO cells.

A. As in Example 2, tumors were grown in six-week-old Balb/C nu/nu male mice injected subcutaneously with a single 0.4 ml dose of 10^7 Clone-26 cells within 40 minutes of harvesting by trypsinization and washing three times with cold serum-containing medium. The cells were kept on ice prior to injection. The animals were sacrificed when the tumors had reached approximately 1.2 cm diameters. The 1.2 cm tumors formed after about 5 weeks.

B. The tumor pieces, 1 mm^3, were implanted by SOI into the left visceral pleura of 8 mice as described by Astoul, P. *et al., Anticancer Research* (1994) 14:85-92; Astoul, P. *J Cell Biochem* (1994) 56:9-15, both incorporated herein by reference. Briefly, the mice were anesthetized by isofluoran inhalation an a small 1 cm transverse incision made on the left lateral chest, via the fourth intercostal space, resulting in total lung collapse. Five tumor pieces were sewn together with a 7-0 nylon surgical suture and fixed by making one knot. The lung was taken up by forceps and the tumor sewn into the lower part of the lung with one suture, after which the lung was returned to the chest cavity and the muscles and skin closed with a single layer of 6-0 silk sutures. The lung was reinflated by withdrawing air from the chest cavity with a 23-gauge needle.

C. Four of the mice were sacrificed at 4 weeks and another 4 at 8 weeks. Pleural tumors for the 4-week group ranged from 244.40 mm^3-522.88 mm^3; those from the 8 week group from 1279.08 mm^3-2714.40 mm^3. This represented mean volumes of 371 mm^3 and 1799 mm^3. Specimens of tissue were sliced at 1 mm thickness and observed directly under fluorescent microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a Leica stereo fluorescence microscope equipped with a mercury lamp power supply and GFP filter sets. All of the animals showed chest wall invasion and local and regional spread of the tumor, but in the 8-week mice, all tumors involved the mediastinum and contralateral pleural cavity as well as metastases on the visceral and parietal pleura.

Pulmonary hilum lymph nodes were involved in 3 of 4 mice of the 4-week group and all of the mice in the 8-week group. Cervical node involvement was detected in one of the mice of the 8-week group, but no other metastases were observed. The animals were also observed directly before the tissues were excised. The margin of the invading tumor in normal lung tissue could be detected by GFP fluorescence and a small vessel could be seen developing at the margin of the tumor.

D. In an additional experiment, 8 nude mice were injected in the tail vein with a single dose of $1x10^7$ Clone-26 cells that had been harvested by trypsinization and washed 3 times with cold serum-containing medium. The injection contained a total volume of 0.8 ml within 40 min. of harvesting. Again, 4 mice were sacrificed at 4 weeks and another 4 at 8 weeks and tissue specimens were obtained and studied by microscopy as described above. Numerous micrometastatic colonies were detected in whole lung tissue in both groups ranging from 5.2 μm to 32.5 μm in the 4-week group and 5.5 μm-178.3 μm in the 8-week group. The colonies from the 8-week group did not appear further developed as compared with those from the 4-week group. Numerous small colonies ranging in number to less than 10 cells were detected at the lung surface in both groups and brain metastases were detected in 1 mouse of the 4-week group and 2 from the 8-week group. One mouse in the 8-week group had systemic metastases in the brain, the submandibular gland, the whole lung, the pancreas, the bilateral adrenal glands, the peritoneum and the pulmonary hilum lymph nodes.

E. In an additional experiment, similar to that set forth in the previous paragraph, the mice injected in a tail vein with 10^7 Clone-26 cells were sacrificed at 4, 8 and 12 weeks and the tissues examined as described. Most of the colonies and mice sacrificed at 8 weeks were not obviously further developed compared with those sacrificed at 4 weeks, but numerous small quantities ranging in number down to less than 10 cells and ranging in size from 5.5 μm-110 μm were detected at the lung surface. At 12 weeks, there were many small metastatic colonies which appeared dormant, although other colonies grew extensively by this time, reaching a size up to

1100 μm, suggesting a heterogeneity of dormant and active tumor colonies in the lung.

Example 4

Growth of Clone-26 Tumor Cells in Histoculture

Six-week old SCID/SCID mice were injected intravenously with a single dose of $7.5x10^7$ Clone-26 cells which had been harvested by trypsinization and washed 3 times with cold serum-containing medium and kept on ice as described above. The cells were injected in a total volume of 0.5 ml within 40 minutes of harvesting. After 3 weeks, numerous micrometastatic colonies were detected in whole lung tissue up to approximately 550 μm. After 5 weeks, the mice were sacrificed and the Clone-26 seeded mouse lungs were removed and histocultured on spun gels using the histoculture methods developed by Leighton, J. *Cancer Res* (1957) 17:929-941; Leighton, J. *et al., Cancer Res* (1960) 20:575-597; Hoffman, R.M. *Cancer Cells* (1991) 3:86-92. Tumor colonies spread rapidly in the lung tissue over time and after 1 week the tumor cells started to invade and colonize supporting collagen sponge-gel. After 2 weeks, tumor cells formed satellite colonies in the sponge-gel distant from the primary colonies in the lung tissue, thus growing faster in histoculture than in SCID mice. Tumor colonies could grow in histoculture for more than 1 month.

Example 5

Construction of a Retroviral Expression Vector for GFP and Preparation of Labeled Tumor Cell Lines

Figures 1a and 1b show the construction of expression vectors for GFP under control of the SV40 promoter. The constructs employ commercially available pEGFP series vectors available from Clontech. Both bacterial and mammalian expression vectors are available which permit production of additional proteins, as well as GFP, either as fusions or in dicistronic systems. Figure 1a shows the construction of an expression vector, pGFP/Met, for a fusion of GFP with methioninase; Figure 1b shows the construction of a vector pGFP/SV40 for production of a fusion protein of GFP with the SV40 T-antigen.

Commercial vectors containing the GFP coding sequence of the desired spectral characteristics using the pLEIN system described in Example 6 were transfected into cell lines originating from tumors, such as human breast cancer, human prostate cancer, human glioblastoma and mouse melanoma. In this manner, human breast cancer cell lines MF-7, MDA-MB468 and MDA-MB435, human prostate cancer cell lines PC3 and DU145, human glioblastoma cell line 324, human lung cancer cells Anip-73 and H460, human colon cancer cells lines Colo-205, HCT-15 and WiDr, human gastric cancer cell line NVGC-4, human kidney cancer cell line SN12C, human tongue cancer cell line SCC-25, human melanomas LOX and SK-mel-5, labeled Chinese hamster ovary cells from cell line CHO-K1 and mouse melanoma cell line B16 labeled with green fluorescent protein were established.

The SV40 T-antigen protein is useful to immortalize cultured cells so as to establish permanent cell lines. Accordingly, the vector pGFP/SV40 is transfected into a series of tumor cell cultures to provide fluorescent immortalized cell lines.

Example 6

In Vivo Labeling of Established Tumors

Unlabeled tumors derived from the human lung cancer cell line Anip973 were established in mice using the procedure set forth in Example 3, paragraphs A and B, but substituting unlabeled Anip973 cells for clone 26. The mice were then injected with $1x10^7$ packaging cells containing the retroviral vector GFP-retrovirus pLEIN contained in PT67 cells. This virus packaging system is available from Clontech, San Diego, California. pLEIN contains an insert of the coding sequence for EGFP, a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. It has an excitation maximum of 488 nm and an emission maximum at 507 nm. This mutant contains a double amino acid substitution at position 64 from Phe to Leu and at position 65 from Ser to Thr. It is described by Comack, B. *et al. Gene* (1996) 173:31-38. There are more than 190 silent base changes to maximize human codon usage preferences as described by Haas, J. *et al. Curr Biol* (1996) 6:315-324. Thus, pLEIN contains the above-described GFP coding sequence inserted into the multiple cloning site of pLXIN to obtain a

dicistronic expression system which permits coordinated translation of the GFP and neomycin resistance. Three days after injection of the cells into the peritoneal cavity of the mice, the tumor cells could be seen in the seminal vesicles under bright-field microscopy and under fluorescent microscopy.

Example 7

Observation of Angiogenesis

A suspension containing $1x10^7$ clone-38 cells, described in Example 1, were injected into the peritoneal cavity of a mouse. Five days later, the mouse was injected in the tail with rhodamine and the mouse was then put under anesthesia and the abdominal cavity opened sufficiently to visualize the tumor. Recovery from this surgery is straightforward. In some cases, abdominal opening is unnecessary as the intraperitoneal tumors can be visualized through intact skin. Tumors were visible in the abdominal cavity and angiogenesis was apparent as identified by the rhodamine fluorescence. Similar results were found in tumors growing in the omentum in the wall of the small intestine, and in the mesentery.

In an analogous experiment, a suspension containing $1x10^7$ cells of clone-26, described in Example 1, were injected into the peritoneal cavity of a mouse. After one day, tumors appeared in the mesentery and in the colon wall. These were observed by anesthetizing the mouse and a minimal opening of the abdomen. Observations on day 3 of a similarly treated mouse showed tumors in the wall of the small intestine and in the omentum as well as in the colon wall and mesentery. On day 5, a similarly treated mouse was injected in the tail with 100 µl of $2x10^{-3}$ M rhodamine and a few vessels could be seen in the tumor growing in the mesentery. After day 60, numerous vessels were seen in the tumor growing in the colon wall.

Claims

1. A method to follow the progression of metastasis of a primary tumor, which method comprises optionally opening a vertebrate subject which has been modified to contain a tumor that expresses green fluorescent protein (GFP) and observing the tissues for the presence of fluorescence.

2. The method of claim 1 wherein the subject is opened.

3. The method of claim 2 wherein at least some of the tissues are excised.

4. The method of claim 3 wherein said excised tissues are observed by microscopic examination.

5. The method of any of claims 1-4 wherein said vertebrate subject has been modified to contain a tumor that expresses GFP by surgical orthotopic implantation of said tumor.

6. The method of any of claims 1-4 wherein a primary tumor endogenous to said subject has been modified to contain an expression system for GFP.

7. The method of claim 6 wherein said expression system comprises a viral promoter; and/or

wherein said vertebrate subject is human; and/or

wherein said expression system further comprises a nucleotide sequence encoding a therapeutic protein.

8. A vertebrate subject which has been modified to contain a tumor expressing GFP.

9. The vertebrate subject of claim 8 which is a mammal; and/or

wherein said subject is immunocompromised; and/or

wherein said tumor is a lung tumor or an ovarian tumor; and/or

wherein said GFP is hGFP-S65T; and/or

wherein either said tumor expressing GFP is provided by surgically implanting a precursor tumor expressing GFP at a tissue or organ site said precursor tumor comprising cells from which cells contained in the tumor are derived or said tumor expressing GFP is provided by supplying an expression system for said GFP to a tumor endogenous to said subject.

10. Tumor cells modified to produce GFP, which tumor cells have been transfected with an expression vector containing a nucleotide sequence encoding said GFP under control of heterologous regulatory sequences.

11. The cells of claim 10 which are an immortalized cell line.

12. The cell line of claim 11 which is a human breast cancer cell line, a human prostate cancer cell line, a human glioblastoma cell line, a human lung cancer cell line, a human colon cancer cell line, a human gastric cancer cell line, a human kidney cancer cell line, a human tongue cancer cell line, a human melanoma cell line, a mouse melanoma cell line or a Chinese hamster ovary cell line.

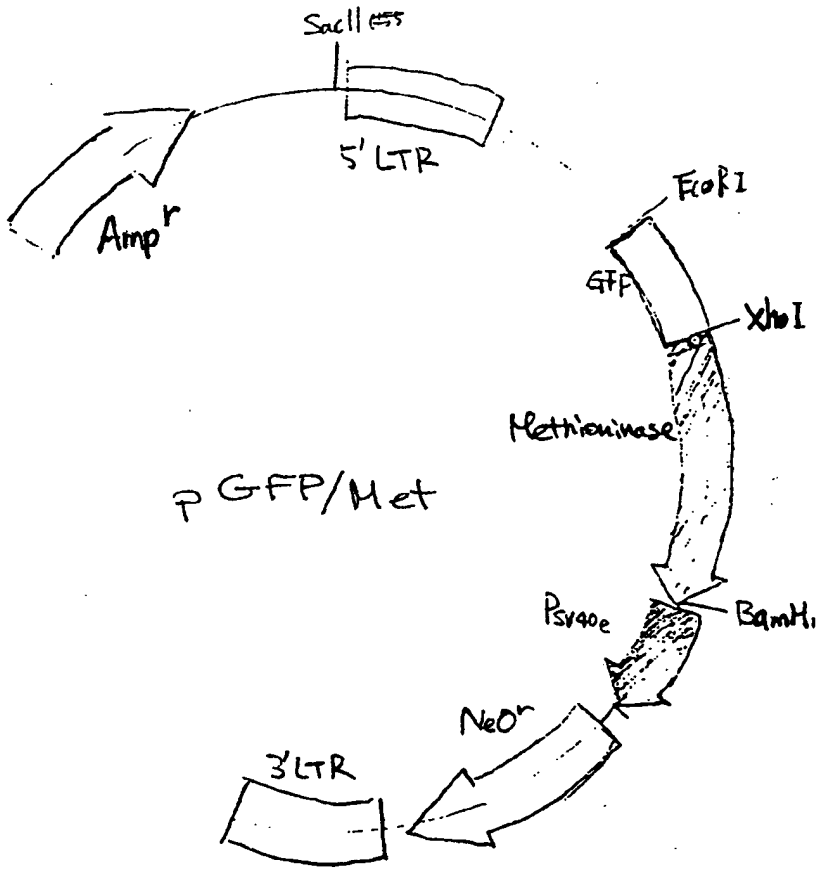
13. Tumor cells modified to produce GFP, which tumor cells have been transfected with an expression vector containing a nucleotide sequence encoding said GFP and a nucleotide sequence encoding a therapeutic protein or an immortalizing protein.

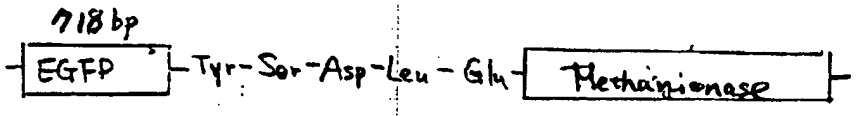
14. A method to prepare a vertebrate which harbors a tumor expressing GFP which method comprises subcutaneously administering to a vertebrate subject an amount of the tumor cells of claim 10 sufficient to effect production of a tumor in said subject, or which method comprises introducing a retroviral expression system for said GFP into said subject.

15. A vertebrate tissue containing metastatic tumor cells wherein said tumor cells produce GFP, or a histoculture prepared from said tissue.

16. A method to observe angiogenesis in tumors contained in a vertebrate subject, which method comprises optionally exposing tissues of said subject and observing blood vessels in said tumors, wherein said blood vessels are visualized by the presence of a contrast dye and said tumor has been modified to express GFP.

17. An immortalized cell line wherein cells which represent said cell line have been modified to contain SV40 T-antigen and visible amounts of GFP.


SacII
5' LTR
Amp^r
EcoRI
GFP
XhoI
Methionase
pGFP/Met
Psv40e
BamHI
NeO^r
3'LTR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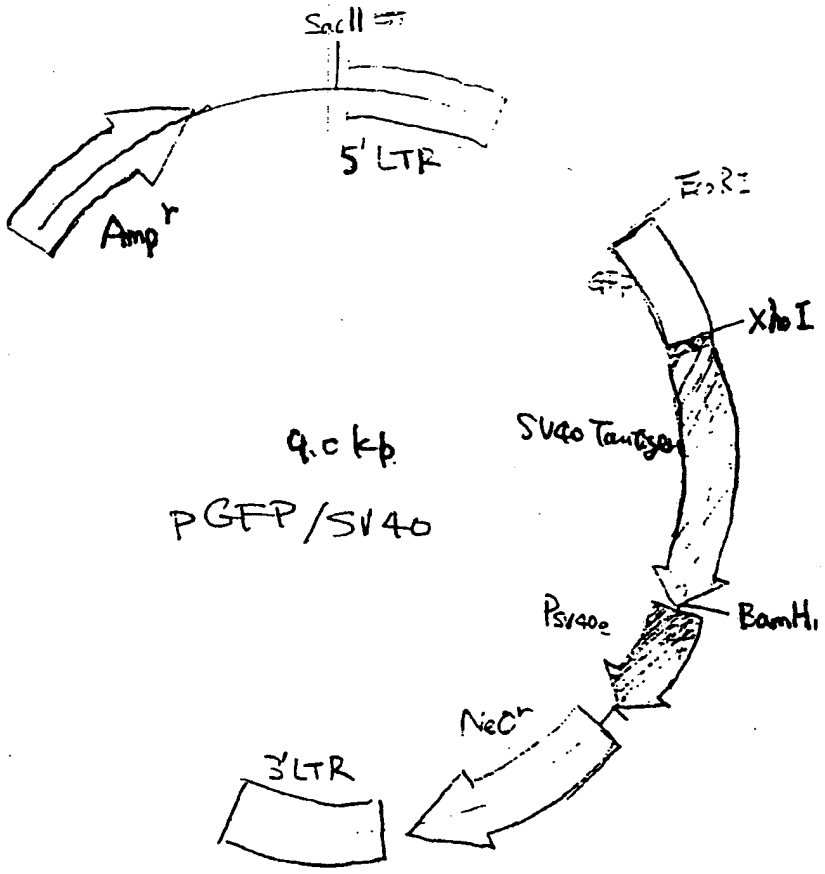
EGFP
Tyr-Ser-Asp-Leu-Glu
Methainionase

Fig 1a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5' LTR
Amp r
EcoRI
GFP
XhoI
SV40 Tantigen
9.0 kb
pGFP/SV40
PSV40e
BamHI
NeoR
3'LTR

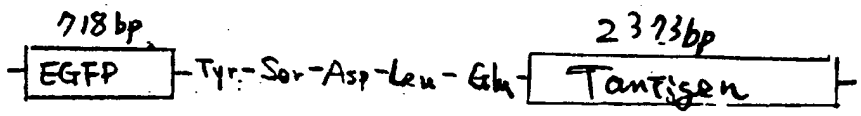
718 bp
EGFP
Tyr-Ser-Asp-Leu-Glu
2373bp
Tantigen

Fig 1b

INTERNATIONAL SEARCH REPORT

Inter. .onal Application No
PCT/US 98/08457

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/86 C12N5/10 A01K67/027 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEVY J P ET AL: "RETROVIRAL TRANSFER AND EXPRESSION OF A HUMANIZED, RED-SHIFTED GREEN FLUORESCENT PROTEIN GENE INTO HUMAN TUMOR CELLS" NATURE BIOTECHNOLOGY., vol. 14, no. 5, May 1996, pages 610-614, XP000673366 PUBLISHING US cited in the application see page 611, column 1, paragraph 1	10-12
Y	see page 613, column 1, line 11 - line 21	1,6-9,13
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 1998	12/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

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INTERNATIONAL SEARCH REPORT

Inten onal Application No
PCT/US 98/08457

C:(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PLAUTZ, J. D. ET AL.: "Green fluorescent protein and its derivatives as versatile markers for gene expression in living Drosophila melanogaster, plant and mammalian cells" GENE, vol. 173, 1996, pages 83-87, XP002073529 AMSTERDAM NL see page 86, line 2 - line 8 ---	10,11
Y	CHALFIE M ET AL: "GREEN FLUORESCENT PROTEIN AS A MARKER FOR GENE EXPRESSION" SCIENCE, vol. 263, 11 February 1994, pages 802-805, XP002003599 see page 803, column 3, paragraph 3 ---	1-16
Y	LIN, W.C. ET AL.: "Bacterial lacZ gene as a highly sensitive marker to detect micrometastasis formation during tumor progression" CANCER RESEARCH, vol. 50, no. 9, 1 May 1990, pages 2808-2817, XP002078058 cited in the application see the whole document ---	1-16
Y	LIN, W.C. & CULP, L. A.: "Altered establishment/clearance mechanisms during experimental micrometastasis with live and/or disabled bacterial lacZ-tagged tumor cells" INVASION METASTASIS, vol. 12, May 1992 - August 1992, XP002078059 released febrary 1993 see page 207, column 1, line 26 - line 30 ---	1-16
P,X	CHISHISMA, T. ET AL.: "Metastatic patterns of lung cancer vizualized live and in process by green fluorescence protein expression" CLINICAL AND EXPERIMENTAL METASTASIS, vol. 15, no. 5, September 1997, pages 547-552, XP002078061 cited in the application see the whole document --- -/--	1-5, 7-12,14, 15

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 98/08457

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	CHISHIMA, T. ET AL.: "Visualization of the metastatic process by green fluorescent protein expression" ANTICANCER RESEARCH, vol. 17, no. 4a, July 1997, pages 2377-2384, XP002078062 cited in the application see the whole document	1-5, 7-12,14, 15
P,X	CHISHIMA, T. ET AL.: "Cancer invasion and micrometastasis visualized in live tissue by green fluorescent protein expression" CANCER RESEARCH., vol. 57, no. 10, 15 May 1997, pages 2042-2047, XP002078063 MD US cited in the application see the whole document	1-5, 7-12, 14-16
P,X	CHISHIMA, T. ET AL.: "Governing step of metastasis visualized in vitro" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 94, no. 21, October 1997, pages 11573-11576, XP002078064 WASHINGTON US cited in the application see the whole document	1-4, 8-13,15
P,X	HYER, M.L. ET AL.: "Adenovirus-mediated gene transfer of GFP (green fluorescent protein) to mouse and human prostate cancer cells" CANCER GENE THERAPY, vol. 4, no. 6, November 1997, pages s29-s30, XP002078065 see the whole document	1-4, 6-10,13, 15
P,X	WO 97 45550 A (BAXTER INT ;ZHANG WEI WEI (US); ALEMANY RAMON (US); DAI YIFAN (US)) 4 December 1997 see page 84, line 4 - page 89, line 13; figure 8	8-10,13
P,Y	LI Y & HORWITZ, M.S.: ""USE OF A GREEN FLUORESCENT PROTEIN IN STUDIES OF APOPTOSIS OF TRANSFECTED CELLLS"" BIOTECHNIQUES, vol. 23, no. 6, December 1997, pages 1026-1029, XP002068179 see the whole document	1-16

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/08457

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-3, 5-7 and 16 are directed to an in vivo diagnostic method and claims 1-3, 5-7 and 14 are directed to a surgical method, in both cases practiced on the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ıal Application No
PCT/US 98/08457

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9745550 A	04-12-1997	NONE	